

Nicotine enhances invasion and metastasis of human colorectal cancer cells through the nicotinic acetylcholine receptor downstream p38 MAPK signaling pathway

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Abstract. Nicotine as a cigarette component is an established risk factor for colorectal cancer tumorigenesis. The downstream signaling pathways of nicotinic acetylcholine receptors (nAChRs) are believed to be responsible for the cellular effects. In the present study, we evaluated the effects and novel mechanisms for nicotine on the capacity for colorectal cancer cell invasion and metastasis. LOVO and SW620 colorectal cancer cells were stimulated with nicotine *in vitro*. A Transwell chamber model was applied to detect the capacity for tumor cell invasion. Assays for gelatin zymography and western blotting were applied to detect the activity and expression of metastasis-related matrix metalloproteinases (MMPs), respectively. Signal transduction was assessed by immunoblotting for the phosphorylation of relevant signal molecules and the application of pharmaceutical inhibitors. We showed that nicotine increased LOVO and SW620 colorectal cancer cell invasion along with enhanced activity and expression of MMP-1, -2 and -9. Nicotine increased phosphorylation of p38, ERK, Akt and PI3K p85 but had no effect

on phosphorylation of JNK, or NF- κ B. Of the pharmaceutical inhibitors of U0126 (ERK1/2 inhibitor), LY294002 (Akt activation inhibitor), SB239063 (p38 MAPK activation inhibitor) and hexamethonium (Hex) (nAChRs inhibitor), the cellular and molecular effects were reduced by the applications of SB239063 and Hex. We concluded that nicotine stimulates the invasion and metastasis of colon cancer cells *in vitro* via activation of the nAChRs and the p38 MAPK downstream signaling pathway. Therefore, p38 MAPK may have potential as a therapeutic target for smoking-related human colorectal cancer metastasis.

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide and is the second leading cause of cancer-related mortality in the developed countries (1). Hepatic metastasis (e.g., liver metastasis) as an ominous event in the natural history and progression of CRC contributing to the major cause of mortality in CRC patients (2,3).

Cancer metastasis is a multistep process by which cancer cells disseminate from primary tumors and establish secondary lesions in distant organs (4). Invasive migration and proteolytic extracellular matrix (ECM) remodelling are believed to be independent processes that control cancer cell invasion (5). Matrix metalloproteinases (MMPs) are members of pericellular proteases that are able to degrade ECM components (6). Upregulation of MMP-2 and MMP-9 was reported to accelerate cell migration and invasion in CRC (7). In particular, results from two recent high throughput-based investigations also pointed to the vital roles of MMPs in CRC invasion and metastasis (8,9).

Nicotine is one of the active ingredients and the major addictive component of cigarette smoking. By binding and activating of nicotinic acetylcholine receptors (nAChRs), nicotine can activate several signalling pathways. The link between nicotine to colon cancer tumorigenesis, i.e. cell proliferation and angiogenesis have been extensively studied (10). Besides, there is also evidence showing that nicotine can enhance colon cancer cell migration by induction of fibronectin (11,12). Nevertheless, the effects and novel mechanisms

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Abbreviations: nAChR, nicotinic acetylcholine receptor; CRC, colorectal cancer; MMPs, matrix metalloproteinases; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; PI3K, phosphatidylinositol 3-kinase

Key words: nicotine, colorectal cancer, invasion and metastasis, MMPs, p38 MAPK

for nicotine on the capacities for colon cancer cell invasion and metastasis are not fully illustrated yet. In the present study, using LOVO and SW620 colon cancer cells grown *in vitro*, we revealed that nicotine increased the cancer cells invasion along with enhanced activities and expressions of metastasis-related MMP-1, -2 and -9. We also proved that the bioactivities of nicotine we observed were depended on activations of the nAChRs and downstream p38 MAPK signaling pathway. Taken together, we reported that nicotine enhances invasion and metastasis of human CRC cells through nAChRs downstream p38 MAPK signaling pathway. Therefore, p38 MAPK may serve as a novel therapeutic target for smoking-related human CRC metastasis.

Materials and methods

Materials. Cell culture RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen (Shanghai, China); antibodies for MMP-1, MMP-2, MMP-9 and MMP-10 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); antibodies for phospho-ERK1/2, phospho-Akt (Ser473), phospho-JNK, phospho-p38, phospho-p65 (NF- κ B), phospho-PI3K (55/85), ERK1/2, Akt, JNK, p38 and p65NF- κ B were purchased from Cell Signaling Technology (Beverly, MA, USA); antibody for β -actin was purchased from Sigma-Aldrich (St. Louis, MO, USA); inhibitors of U0126, LY294002 and SB239063 were purchased from Beyotime Institute of Biotechnology (Shanghai, China); Hexamethonium (Hex) bromide was from Sigma-Aldrich (Shanghai, China). Goat anti-rabbit IgG antibody conjugated to horseradish peroxidase and goat anti-rat IgG antibody conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology, Inc.

Cell culture. The LOVO and SW620 human CRC cells were obtained from the American Type Culture Collection (ATCC; USA). The cells were grown in RPMI-1640 medium containing 10% of FBS, glutamine and antibiotics (penicillin/streptomycin), and were maintained in a humidified incubator at 37°C with a supply of 5% CO₂/95% air atmosphere.

Invasion assay. Cell invasion assays were carried out using modified Boyden chambers consisting of Transwell (8- μ m pore size; Corning Costar Corp., Cambridge, MA, USA) membrane filter inserts into 24-well tissue culture plates. In brief, the upper surfaces of the membranes were coated with 50 μ l Matrigel (BD Biosciences, San Jose, CA, USA) 37°C for 6 h. SW620 or LOVO cells (1.0×10^5) in 200 μ l serum-free RPMI-1640 were added to each Transwell chamber supplied with or without nicotine or/and inhibitors as indicated. Cell culture media with 20% of FBS were added in the lower chamber. Cells were allowed to invade toward the underside of the membrane under cell culture condition for 48 h before the non-invading cells were removed by wiping the upper side of the membrane with a cotton swab and the invaded cells were fixed with ice-cold methanol, then the inserts were stained with 0.1% crystal violet in 20% ethanol for 30 min. The cells that invaded to the underside of the membrane were quantitated by cell counting under the light microscopy in five predetermined fields at a magnification of $\times 100$.

Gelatin zymography assay for MMP-2 and -9 activities. The gelatin zymography assay was used to determine the activities of MMP-2 and MMP-9. Cell culture supernatants were collected and concentrated in Amicon Ultra-4 Centrifugal Filter Devices before loading with SDS sample buffer and electrophoresis on a 10% SDS polyacrylamide gel polymerized with 5 mg/ml gelatin. After electrophoresis, the gels were renatured by soaking for 30 min at room temperature in 2.5% Triton X-100. To visualize the bands, the gels were incubated in a developing buffer [50 mM Tris-HCl buffer (pH 7.4), 10 mM CaCl₂] overnight at 37°C before they were stained with 0.5% Coomassie brilliant blue R-250 dissolved in 10% acetic acid and 30% carbinol, and were destained in the washing solution without dye. Gelatinolytic bands were observed as clear zones against the blue background.

Western blotting. Total cellular protein was prepared by lysing the cells with 1xRIPA buffer (CST, Danvers, MA, USA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was determined by the Bradford protein assay. A total of 40 μ g protein lysates from each sample were separated in 10% SDS-PAGE, and were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were incubated overnight on ice with primary antibodies against MMP-1, MMP-2, MMP-9, MMP-10, phospho-ERK1/2, phospho-Akt, phospho-JNK, phospho-p38, phospho-p65 (NF- κ B), phospho-PI3K (55/85), ERK1/2, Akt, JNK, p38, p65 (NF- κ B) and β -actin (all with 1:1,000 dilution). After washing, the membranes were probed with a horseradish peroxidase-conjugated goat anti-rabbit or anti-rat secondary antibody followed detection of signals with the FluorChem E system (Protein Simple, Santa Clara, CA, USA).

Statistical analysis. All assays were performed in triplicate, and experiments were repeated at least three times. Data are presented as the means \pm SEM. Significant differences between two groups were determined by Student's t-test with significance set at $p < 0.05$.

Results

Nicotine promotes LOVO and SW620 human CRC cells invasion in vitro. Firstly, we investigated whether nicotine affects the invasion of CRC cells in a Transwell assay and BD Matrigel™ was used to imitate the ECM. LOVO and SW620 colon cancer cells were grown in the upper chamber in serum-free media without or with the supplement of nicotine in a final concentration of 0.1, 1 and 10 μ M, respectively (11-14). Results showed that both starved LOVO and SW620 cells were able to invade through the Matrigel with the attracting of serum rich media in the lower chamber. Certain numbers of invaded cells stained in blue were counted after 48 h (Fig. 1, controls). The presence of nicotine increased the number of the invaded cells in a dose-dependent manner, and for both of the LOVO and SW620 cells (Fig. 1).

Nicotine promotes the activities and expressions of MMPs in LOVO and SW620 human CRC cells. Next, we detected the activities and expressions of metastasis-related MMPs

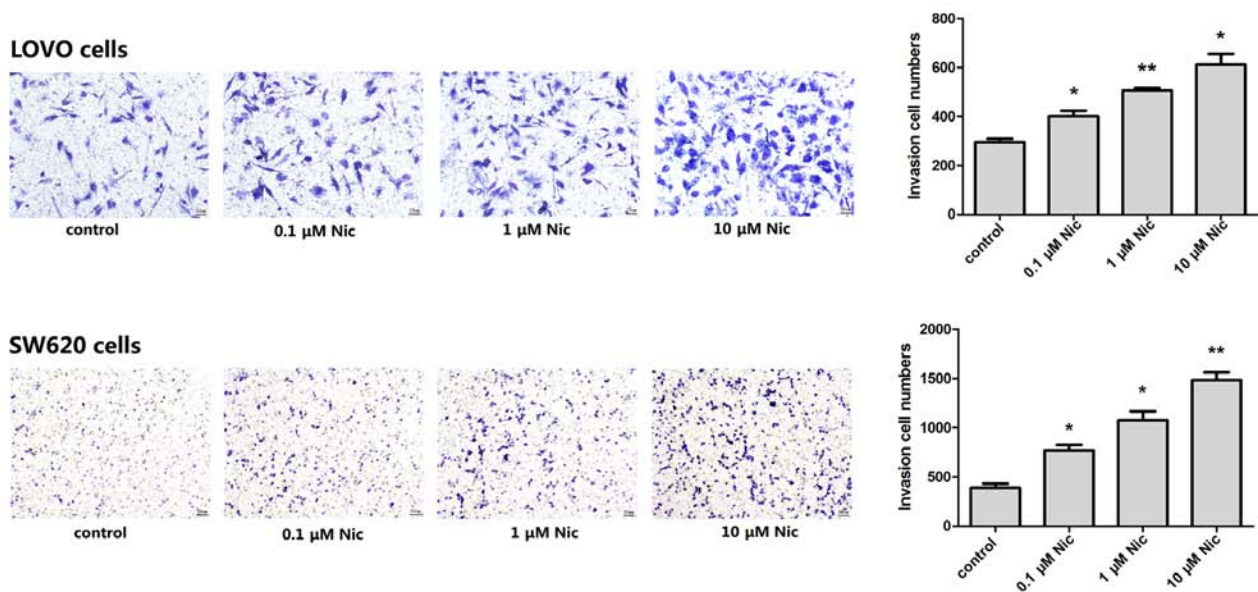


Figure 1. Nicotine promotes LOVO and SW620 human colorectal cancer cell invasion *in vitro*. SW620 or LOVO cells (1.0×10^5) in 200 μ l serum-free RPMI-1640 were added to the upper chamber of Transwell coating with Matrigel and supplied with 0, 0.1, 1 or 10 μ M nicotine, respectively. Cell culture media with 20% of FBS were added in the lower chamber. Three wells were used for each treatment. Forty-eight hours later, the cells invaded to the underside of the membrane were quantitated by cell counting under light microscopy in five predetermined fields at a magnification of $\times 100$. Representative image showing 1 of 5 fields captured from each well. The invasion assay was performed three times independently. * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control. Nic, nicotine.

for LOVO and SW620 human CRC cells without or with the presence of nicotine. A gelatin zymography assay was applied to detect the activity for secreted MMP-9 and -2. In brief, LOVO and SW620 human CRC cells were grown in serum-free medium containing various concentrations of nicotine as indicated above, 24 h before the cell culture supernatants were collected and concentrated for gelatin zymography assay. As indicated in Fig. 2A, there were gelatinolytic bands for MMP-9 and MMP-2, respectively. The nicotine treatments deepen the bands for both MMPs, and in both cell lines.

In a similar experimental setting, western blot assays were applied to detect the expression of a panel of MMPs including MMP-1, -2, -9 and -10 in LOVO cells. As shown in Fig. 2B, the nicotine treatments increased the expression of MMP-1, -2 and -9, but had no effect on MMP-10.

Nicotine activates number of signal transduction pathways in LOVO cells. We investigated the activation of relevant intracellular MAPK (JNK, p38, ERK), NF- κ B and PI3K/Akt signaling pathways caused by nicotine stimulation. To this end, serum-starved LOVO cells were stimulated without or with 10 μ M nicotine for 15, 30, 60 and 120 min, respectively, and then were subjected to western blot assays examining the phosphorylation of JNK, p38, ERK, NF- κ B (p65), Akt and PI3K (p55/p85). As shown in Fig. 3, with total amount of protein was the same, the presence of nicotine brought up the phosphorylation levels of p38, ERK, Akt and PI3Kp55, but did not change that of JNK and NF- κ B (p65).

Stimulative effect of nicotine on MMPs involves nAChRs and p38 MAPK in LOVO cells. Having shown that nicotine stimulates the activation of MAPK/ERK, MAPK/p38 and PI3K (p55)/Akt signaling pathways, we then tried to dissect

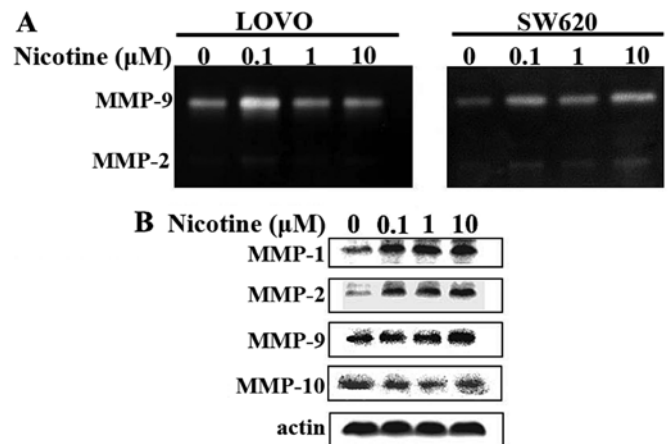


Figure 2. Nicotine promotes the activities and expression of MMPs in LOVO and SW620 human colorectal cancer cells. LOVO and SW620 cells (1.0×10^5) were grown in serum-free medium containing 0, 0.1, 1 or 10 μ M nicotine for 24 h before the cell culture supernatants were collected and concentrated for gelatin zymography assay to determine the activity of MMPs and proteins and extracted for western blotting to determine the expression of MMPs. (A) Nicotine enhances the MMP-9 and MMP-2 enzymatic activity in LOVO and SW620 cells. (B) Nicotine promotes the expression of the MMP-1, MMP-2 and MMP-9 but had no effect on MMP-10 in LOVO cells. Results are representative of three independent experiments.

their relationship to the observed bioactivities for nicotine in regards of stimulating metastasis related MMPs expression in colon cancer cells. To this end, a panel of pharmaceutical inhibitors was applied before the cells were exposed to nicotine stimulation. The activities and expressions of MMPs were assayed by both gelatin zymography and western blotting. In addition, we also included Hex, a pharmaceutical inhibitor for nAChR in the experimental setting to illustrate the role for receptor binding of nicotine.

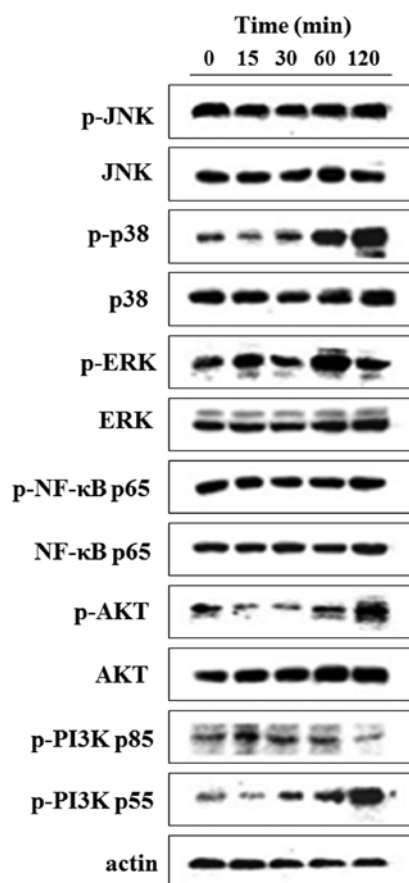


Figure 3. Nicotine activates number of signal transduction pathways in LOVO cells. Serum-starved LOVO cells (1.0×10^5) were incubated with or without $10 \mu\text{M}$ nicotine for 15, 30, 60 and 120 min, and then cells were harvested and cell lysates ($40 \mu\text{g}$ of protein) were subjected to western blotting with antibodies against p-JNK, JNK, p-p38, p38, p-ERK, ERK, p-NF- κB p65, NF- κB p65, p-AKT, AKT, p-PI3Kp85, p-PI3Kp55 and β -actin, respectively. Results are representative of three independent experiments.

As detailed in Fig. 4A, western blot results revealed that the pre-incubation of cells with the p38 MAPK inhibitor SB239063 and nAChR antagonist Hex, but not selective MAPK/ERK inhibitor U0126 and the PI3Ks inhibitor LY294002 attenuated the stimulative effect of nicotine for MMP-1, -2 and -9 expression levels. Similar results are also shown in Fig. 4B, as the gelatin zymography assay result revealed that the SB239063 and Hex attenuated the stimulative effect of nicotine on the activation of MMP-2 and -9.

p38 MAPK are required for the stimulative effect of nicotine on LOVO cell invasion in vitro. We further examined whether p38 MAPK also was affected by stimulative effect of nicotine on LOVO cell invasion *in vitro*. In this regards, we performed the Transwell chamber invasion assay as described above with an additional step of pre-incubation of the cells with SB239063. Results showed that compared to nicotine-treated cells, the application of SB239063 indeed decreased the number of invaded cells ($p < 0.05$), almost down to control levels, i.e. without nicotine treatment (Fig. 5).

Nicotine activation is through nAChRs and downstream of the p38 MAPK signaling pathway in LOVO cells. Finally, we addressed the question whether activation of p38 MAPK

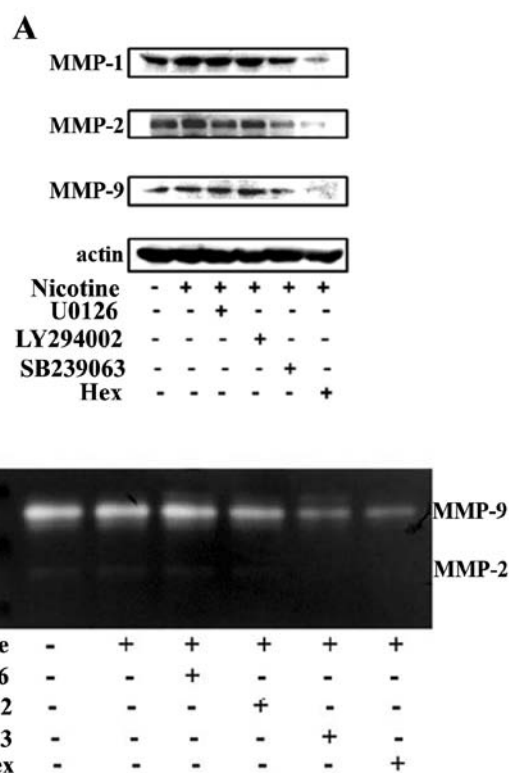


Figure 4. The stimulative effect of nicotine on MMPs involves the nAChRs and p38 MAPK in LOVO cells. LOVO cells (1.0×10^5) were pretreated with $1 \mu\text{M}$ U0126 (ERK1/2 activation inhibitor), $1 \mu\text{M}$ LY294002 (Akt activation inhibitor), $1 \mu\text{M}$ SB239063 (p38 MAPK inhibitor) or $1 \mu\text{M}$ Hex (nicotinic acetylcholine receptors inhibitor) for 1 h followed by the administration of $10 \mu\text{M}$ nicotine for 24 h. Then, the proteins were harvested for western blotting (A), and the cell culture supernatants were collected and concentrated for gelatin zymography assay (B). Results are representative of three independent experiments.

in LOVO cells is indeed dependent on the activation of nAChRs complex. To this end, LOVO cells were pre-treated with SB239063 or Hex for 1 h, respectively, before cells were exposed to nicotine stimulation for 24 h and subsequently subjected to western blotting. As shown in Fig. 6, the application of Hex abolished the enhanced p38 phosphorylation caused by nicotine stimulation, to an extent similar to SB239063, the known p38 MAPK inhibitor treatment.

Discussion

Sufficient evidence has revealed the association of pharmaceutical stimulation of nicotine with tumor cell metastatic dissemination. In mouse tumor transplantation models, nicotine increases the growth and metastasis of various tumor cells including the liver metastasis of orthotopically implanted pancreatic adenocarcinoma cells, subcutaneously injected head and neck squamous cell carcinoma cell lines and NNK-induced lung tumors (15-17). In particular, Wei *et al* showed that nicotine enhanced colon cancer cell migration through activation of $\alpha 7$ nicotinic acetylcholine receptor (nAChR) and induction of E-cadherin (12). In the present study, the stimulatory effect on human colon cancer cell invasion could also be observed after nicotine treatment in a dose-dependent manner ranging from 0.1 to $10 \mu\text{M}$ after 48 h. These concentrations of nicotine

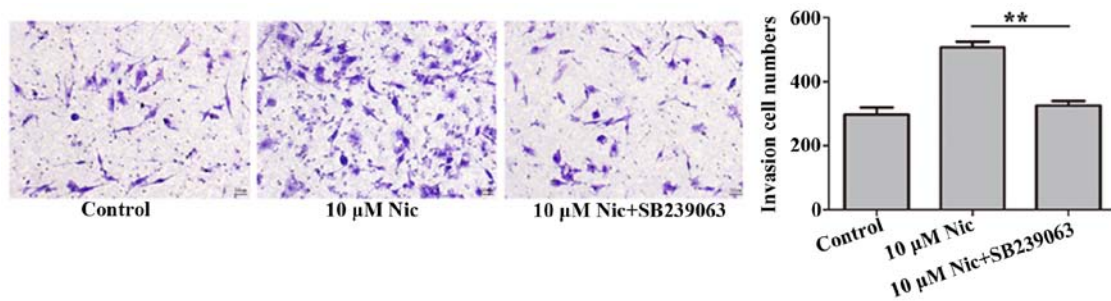


Figure 5. p38 MAPK is required for the stimulative effect of nicotine on LOVO cell invasion *in vitro*. LOVO cells (1.0×10^5) in 200 μ l serum-free RPMI-1640 were added to the upper chamber of Transwell coating with Matrigel and supplied 10 μ M nicotine or nicotine with 1 μ M SB239063. Cell culture media with 20% of FBS were added in the lower chamber. Three wells were used for each treatment. Forty-eight hours later, the cells invaded to the underside of the membrane were quantitated by cell counting under light microscopy in five predetermined fields at a magnification of $\times 100$. Representative image showing 1 of 5 fields captured from each well. The invasion assay was performed three times independently. ** $p < 0.01$. Nic, nicotine.

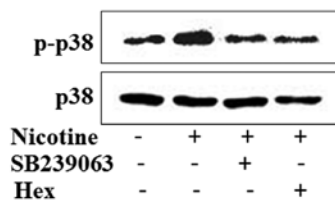


Figure 6. Nicotine activates through nAChRs and downstream p38 MAPK signaling pathway in LOVO cells. LOVO cells (1.0×10^5) were stimulated with SB239063 (1 μ M) or Hex (1 μ M) for 1 h followed by the treatment with 10 μ M nicotine for 24 h, then cells were harvested and cell lysates (40 μ g of protein) were subjected to western blotting with antibodies against p-p38, p38 and β -actin. Results are representative of three independent experiments.

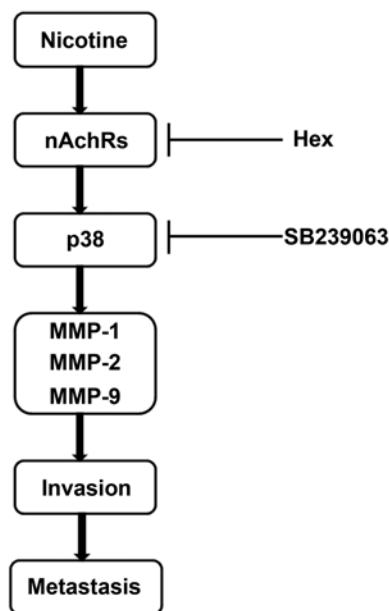


Figure 7. A schematic representation showing that p38 MAPK mediates nicotine enhanced expression of MMP-1, -2 and -9, and the consequent cell invasion of human colorectal cancer cells.

were similar to the amount of nicotine intake in cigarette smokers (18). Therefore, nicotine does affect colorectal cancer (CRC) tumor cell invasion and migration.

Matrix metalloproteinases (MMPs) are members of pericellular proteases that are able to degrade proteolytic extracellular

matrix (ECM) components (6). Despite abundant evidence showing that overexpression of MMPs correlates with tumor aggressiveness and metastatic potential in various cancers such as ovarian, lung, prostate, breast and pancreatic, the role of nicotine in this scenario is not fully understood. There is only one previous study revealing that nicotine contributes to pancreatic ductal adenocarcinoma (PDA) metastasis by inducing MMP9 among others (19). In the present study, we showed that nicotine indeed increased LOVO and SW620 CRC cell invasion along with enhanced activity and expression of MMP-1, -2 and -9. We believe that nicotine directly induced this effect via the activation of nAChRs since the application of Hex, the pharmaceutical nAChRs inhibitor abolished the increased expression of the MMPs.

Little is known concerning the molecular mechanisms by which nicotine promoted tumor development, particularly on the metastatic process of CRC. In other cancer cells, nicotine has been shown to affect various signaling cascades that initiated by the binding of its receptor. In breast cancer cells, nicotine was shown to promote cell migration through a signaling cascade involving protein kinase C (PKC) activation and its downstream effector cdc42 (20). In PDA cells, nicotine was shown to induce the expression of pro-metastasis and pro-angiogenesis osteopontin (OPN) through nAChR and downstream ERK1/2-dependent pathway (21,22). Additionally, activation of the EGFR and downstream AKT and ERK pathways was shown related to the enhancement of cell migration of human malignant glioma cells as response to low concentrations of nicotine treatment (23). In the present study, we revealed that stimulation of CRC cells with nicotine resulted in the activation MAPK/ERK, MAPK p38 and PI3K (p55)/Akt signaling cascades but notably only MAPK p38 was responsible for the MMPs related CRC cell invasion and migration. Amongst the panel of pharmaceutical inhibitors, only p38 MAPK inhibitor SB239063, but not selective MAPK/ERK inhibitor U0126 or the PI3K inhibitor LY294002 attenuated the stimulative effect of nicotine on CRC cell invasion and MMP-1, -2 and -9 expression.

In summary, we demonstrated for the first time that nicotine promotes CRC cell invasion by simultaneously upregulating the expression and activity of MMP-1, -2 and -9 through nAChR-mediated p38 MAPK signaling pathway (illustrated in Fig. 7). The p38 MAPK signaling pathway may exert a central

effect in the nicotine-mediated promotion of cell invasion, suggesting that p38 MAPK may be a promising target for the treatment of smoking-related human CRC metastasis.

Acknowledgements

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